Comparative Transcriptome Analysis of Hot Pepper (*Capsicum annuum* L.) Leaf Heterosis by RNA-seq

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Abstract: *Heterosis has been mostly used in hot pepper breeding and production, but the molecular basis of heterosis has not been extensively studied. In this study, comparative transcriptomes analysis of parental lines (D6, D7) and F_1 hybrids (D6×D7 and D7×D6) was performed. A total of 0.6 billion raw reads, and 0.44 billion high-quality reads were obtained after the filtering process. Statistical analysis of genes with presence/deletion variations showed that, there were 1068 (6.20%) and 780 (4.56%) genes in the "single parent express consistent type" in the direct (D6×D7) and reciprocal (D7×D6) F_1 hybrids, respectively. More genes fit into the non-additive expression type in two F_1 hybrids compared to the parents, and less than 8% of the genes belong to the additive expression type. 66.08% in direct and 62.96% in reciprocal F_1 hybrids belong to the epistatic dominance expression pattern. There were more differentially expressed genes (DEGs) between the two parental lines (351) than between the two hybrids (17). The results of gene ontology (GO) analysis showed that there were obvious differences in electron transmission and photorespiration between two F_1 hybrids. GO terms for regulating plant hypersensitive responses, and MAPK pathways were only enriched in the direct hybrid (D6×D7).*

Keywords: *Heterosis, Transcriptome, RNA-seq, Hybrid hot pepper.*

Introduction

Heterosis describes superior performance such as increases in productivity, growth vigor and propagation in a hybrid offspring compared to the average or the highest value of these traits from both parents [23]. Several models including the dominance, over-dominance and epistasis hypotheses have been used to explain the genetic basis of heterosis, and suggested that the contribution of genes is responsible for the vigorous phenotypes of hybrids over parents. In most crop plants, such as maize, rice and wheat, F_1 hybrids were mostly used as an effective tool in plant breeding. The process encompasses steps from the classification of the heterosis, selection of inbreed lines, and measuring the general combining ability and special combining ability between inbred lines. The breeding program is both time consuming and costly. In recent years, the use of molecular tools to predict the heterosis, followed by rapid screening for the super-heterosis combinations has greatly improved the efficiency of hybrid breeding. For this reason, breeders are extremely interested in discovering the mechanisms underlying the heterosis trait [1, 7, 11, 21]. However, knowledge in this area remains deficient even though studies in this field have been ongoing for over hundreds of years [3].

Hot pepper (*Capsicum* spp.), which originated and initially domesticated in the Americas, is an extensively grown spice and comprises a major ingredient in many cuisines around the world [23]. As a member of the family *Solanaceae*, it belongs to a category of plants that are often cross-pollinated. The investigation of the molecular mechanisms of hot pepper heterosis has been ongoing for over 50 years. Recently, studies have focused on phenotypes [12, 24, 25], physiolchemical properties [9], heredity and molecular makers [2, 18, 19, 29, 36]. In recent years, the various molecular marker technologies such as RAPD, ISSR, AFLP, and SSR have been used to classify hot pepper populations and heredity distances, making significant progress in all these fields. However, molecular markers generated from these research projects often gave inconsistent results when they were used in predicting hot pepper heterosis. It is clear that hot pepper heterosis is caused by a highly complex mechanism. Therefore, the appropriate approach to address this issue should integrate phenotypes, physiol-chemical, heredity, genomic and transcriptomic analysis.

There have been several successful cases in using gene expression data to explore plant heterosis using model plant species which include rice [8, 37, 39], maize [4, 14, 17] and *Arabidopsis* [5]. However, none have been reported on hot pepper heterosis. This study uses the public RNA-seq data from two hot pepper genotypes and their reciprocal F_1 hybrids. Results provide the information of candidate genes pathways for breakthrough discoveries in revealing the underlying molecular mechanisms of hot pepper heterosis.

Materials and methods

Plant materials and growing conditions

Four hot pepper genotypes were used in this study, including *Capsicum annuum* L. (D6), *Capsicum annuum* L. (D7), and their reciprocal crosses (D6×D7 and D7×D6). Seeds of the four genotypes were sown at the Baiyun Experimental Station, Vegetable Institute, Guangdong Academdy of Agricultrual Sciences. Four weeks after sowing, the sixth true leaves were sampled, frozen immediately in liquid N_2 and stored at –80 °C until analysis.

RNA-seq data

Total RNA was extracted using a modified CTAB method, and mRNA was purified with Oligo (dT). After shearing into 200 nt fragments, cDNAs were reverse-transcribed using random hexanucleotide primers. After blunt end repair and adaptor ligation, gene fragments were amplified using PCR. The cDNA libraries were sequenced on an Illumina Hiseq 2000, and four libraries were sequenced. The high quality reads were mapped to the referenced genes and six transcriptome expression files were generated. Reads per kb per million reads (PPKM) [16] was used to calculate the level of gene expression. The RPKM method can eliminate the effect of gene length and sequencing depth differences among sequencing experiments. Therefore data of gene expression level can directly be used to identify differentially expressed genes (DEGs) among samples.

Clustering of gene expression types

Gene expression data were fit into the following five putative gene expression types: (1) expressed in the two parents, but not in F_1 (co-expressed genes); (2) expressed in one parent, but not the other parent nor in F_1 (absent in hybrid); (3) expressed in F_1 but not in either parent (expressed in only one parent); (4) expressed in F_1 and one of the two parent (expressed only in hybrid); (5) expressed in both parents and F_1. The former four types of expression belong to the qualitative differential gene expression which is caused by the presence/absence variations (PAV) of the genes, and the fifth type is caused by quantitative difference in gene expression among genotypes.

The second classification method for F_1 hybrids and parents can be described as [25-26]: A = (high parent – F_1)/(high parent – low parent). High parent: (1) A < 0.0, (2) 0.0-0.2; mid parent: (3) 0.2-0.4, (4) 0.4-0.6, (5) 0.6-0.8; low parent: (6) 0.8-1.0, (7) A > 1. High parent and low parent in F_1 each represents high or low expression parent in the hybrids.

Clustering of differentially expressed genes in the two parents

Expression types of genes showing differential expression levels in the two parental lines were clustered using the method described in Table 1.

Table 1. Criteria of gene expression mode in hybrid
(for genes are not equally expressed in two parents)

Category	Over-dominance, (-)	Dominance, (-)	Partial-dominance, (-)	Additive	Partial-dominance, (+)	Dominance, (+)	Over-dominance, (-)
$d/\|a\|$	$(-\infty,-1.2)$	$[-1.2, -0.8)$	$[-0.8, -0.2)$	$[-0.2, 0.2]$	$(0.2, 0.8]$	$(0.8, 1.2]$	$(1.2, +\infty)$

In the Table 1, the clustering criteria were calculated using the equation:

$$d/|a| = (F_1 - \mu)/|P_1 - \mu|,$$

where F_1 is the expression level of F_1 hybrid, μ is the mean of the expression amounts from both parents, and P_1 is the expression level in one parent.

Threshold criteria of differential expression genes

Only genes with a two-fold change or higher difference in expression levels in the pair-wise comparisons (FDR ≤ 0.05) were considered.

Results

Mapping reads to the annotated genome of hot pepper

The RNA-seq analysis of cDNA libraries generated a total of 0.6 billion reads. After filtering and trimming the adaptors, 0.44 billion high quality reads (100 bp) were obtained which were mapped to the *Capsicum annuum* L. (Pepper.v.1.5) reference genome [23] using Tophat [30]. The ratio of alignment is 83.74-85.43%, and the unique alignment ratio is 70.32-79.69%. For all the sequences from the four hot pepper genotypes, 65.82-67.13% reads were aligned to exons, 2.49-2.96% reads aligned to intron regions, and 30.36-31.57% reads mapped to inter-genic regions as shown in Table 2.

The gene presence /deletion variations among F_1 and parents

As shown in Table 3, statistical analysis showed that 15,128 (87.87%) genes were found in the D6×D7. Among these genes, 17,217 genes were also expressed in both parents and hybrids, 212 (1.23%) genes expressed in both parents but not in hybrids (absent in hybrid), 488 (2.83%) expressed in only one parent but in hybrid (expressed in only one parent), 331 (1.92%) genes only expressed in hybrids, not in the parental lines (expressed only in hybrid), 1 068 (6.20%) genes expressed in hybrids and one parental line (expressed in one parent and hybrid).

Table 2. Statistics of alignment results of RNA-seq data

Items	D6	D6×D7	D7	D7×D6
Total reads	14,212,656	15,550,315	16,122,929	13,645,980
Length of reads	50	50	50	50
Bases	723,342,350	785,952,850	818,023,850	693,818,000
Uniquely mapped reads	10,247,469	12,392,084	12,485,849	9,595,259
Percentage of uniquely mapped reads	72.10%	79.69%	77.44%	70.32%
Total filtered reads	14,212,656 (98.24%)	15,550,315 (98.93%)	16,122,929 (98.55%)	13,645,980 (98.34%)
Percentage of exon mapped reads	65.84%	65.87%	65.82%	67.13%
Percentage of intron mapped reads	2.57%	2.96%	2.75%	2.49%
Percentage of intergenenic mapped reads	31.57%	31.15%	31.40%	30.36%

In the reciprocal F_1 (D7×D6), 17,096 genes were expressed, which is 120 fewer genes than D6×D7. Among these genes, 14,927 (87.31%) were also expressed in both parents and hybrids (co-expressed genes), 413 (2.42%) genes only in both parents but not in the hybrids (absent in hybrid), 465 (2.72%) genes expressed in only one of the two parents, but not in the hybrids (expressed in only one parent), 211 (1.23%) genes expressed only in the hybrids, but not in the parental lines (expressed only in hybrid), 780 (4.56%) genes expressed in hybrid and one parental line (expressed in one parent and hybrid).

Table 3. Gene presence/absence variation

Category	Total	Co-expressed genes	Absent in hybrid	Expressed in only one parent	Expressed only in hybrid	Expressed in one parent and hybrid
D6×D7	17,217	15,128 (87.87%)	212 (1.23%)	488 (2.83%)	331 (1.92%)	1068 (6.20%)
D7×D6	17,096	14,927 (87.31%)	413 (2.42%)	465 (2.72%)	211 (1.23%)	780 (4.56%)

For the pair-wise comparison, in the reciprocal F_1 (D7×D6) 17,096 genes were identified, it is 120 fewer genes than D6×D7. In the four genotypes, genes with the presence/deletion variations were clustered under the "expressed only in hybrid" and "expressed in only one parent" which should belong to the dominant model. The enriched biological pathways of these genes should be the focus of studies in the molecular mechanism of hot pepper heterosis.

Differential gene expression in the high, medium and low parental lines

The level of gene expression in the four genotypes should be analyzed as quantitative traits. Based on the relative expression level in the two hybrids and the parents, genes were placed into three major and seven minor groups. According to the distribution scheme, 9,078 genes in D6×D7 and 6,254 genes in reciprocal F_1 (D7×D6) showed higher expressed levels in a hybrid compared to the parents. 3,419 genes in D6×D7 and 3,443 genes in reciprocal F_1 (D7×D6) exhibited mid-parent expression levels in a hybrid. 4,816 genes in D6×D7 and

7,616 genes in reciprocal F_1 (D7×D6) showed low-parent expression levels in a hybrid, as shown in Fig. 1. The χ-axis plots the difference in expression of the hybrid and the high parent divided by the difference in the level of expression between the two parents [(high parent – F_1)/(high parent – low parent)].

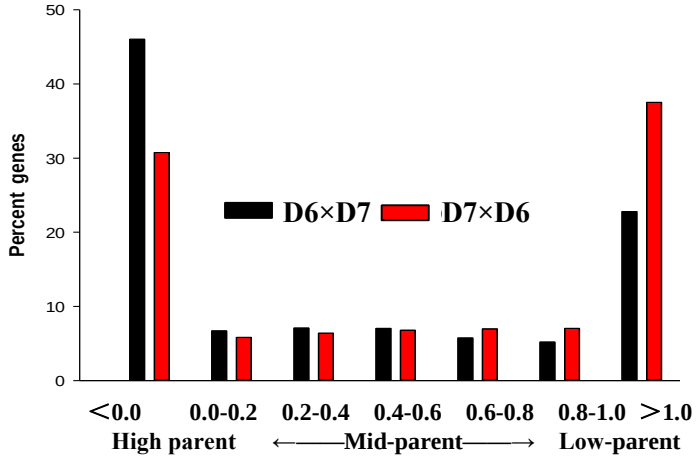


Fig. 1 Frequency distribution of high-parent, mid-parent, and low-parent expression in the level of expression between the two parents

Genes at high expression levels were plotted to the two ends, representing the high and the low parent expression types on the gene expression distribution curve shown in Fig. 1. These results indicate that partial single parent dominance is the major gene expression type, and the additive effect of elite dominance genes in F_1 would have made some contribution to the F_1 heterosis.

Classification of expression types for genes showing differential expression levels in the parental lines

Analysis of the DEGs in the two parental lines indicates that compared to the parents, hybrids (D6×D7 and reciprocal F_1 D7×D6) contain the majority of the genes which follows the non-additive expression patterns, and less than 8% of the genes showing the additive-expression effects, as shown in Table 4. Among all the non-additive expression genes, the majority belongs to the super-dominance expression type, accounting for 66.08% and 62.96% of total number of genes identified in D6×D7 and reciprocal F_1 hybrids. These results concur with conclusions derived from data in Fig. 1. The ratio of dominant genes is rather low at 12.50% and 13.82% in D6×D7 and reciprocal F_1 hybrids. The number of genes in the partial dominance expression group is in the middle range. Among the super-dominance expressed genes, the number of genes belonging to the positive super-dominance is higher than the negative super-dominance expression type in D6×D7 and reciprocal F_1 hybrids.

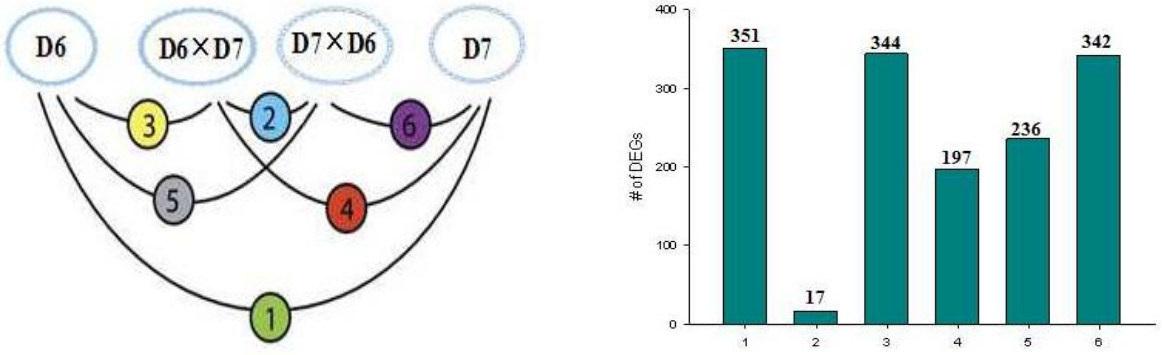
Pair-wise differential gene expression analysis and functional classification

To achieve a comprehensive overview of differential gene expression, all possible (N = 6) pair-wise comparisons of the four genotypes were performed, as shown in Fig. 2A. Putative DEGs were identified using the following criteria: (1) false discovery rate (FDR) less than or equal to 0.05, and (2) fold change (FC) greater than or equal to 2. Using these criteria, 351 DEGs between two parents D6 and D7 were identified, which is much higher than the number 17 of DEGs identified between the two hybrids, D6×D7 and D7×D6, as shown in Fig. 2B. The number of DEGs between parents and hybrids ranged from 197 to 342.

Comparisons between the two parents and their hybrids showed fewer DEGs than the comparison between two parents, but more than the comparison of the reciprocal hybrids, as shown in Fig. 2B. The number of DEGs between F_1 hybrids and the maternal line is higher than that of the paternal line.

Table 4. Criteria of gene expression mode in hybrid
(for genes are not equally in two parents)

Category	$d/\|a\|$	D6×D7	D7×D6
Over-dominance (-)	$(-\infty, -1.2)$	2 373 (17.23%)	4 180 (30.61%)
Dominance (-)	$[-1.2, -0.8)$	723 (5.25%)	1 114 (8.16%)
Partial-dominance (-)	$[-0.8, -0.2)$	1 390 (10.1%)	1 755 (12.85%)
Additive	$[-0.2, 0.2]$	1 152 (7.72%)	1 072 (7.28%)
Partial-dominance (+)	$(0.2, 0.8]$	1 559 (11.32%)	1 416 (10.37%)
Dominance (+)	$(0.8, 1.2]$	998 (7.25%)	773 (5.66%)
Over-dominance (-)	$(1.2, +\infty)$	6 726 (48.85%)	4 417 (32.35%)



A) B)

Fig. 2 Identification of differentially expressed genes in all pair-wise comparisons between four genotypes. (A) All possible pair-wise comparisons between the four genotypes; (B) Number of differentially expressed genes (DGEs) in all possible pair-wise comparison.

Using the gene ontology (GO), DEGs were classified into different functional groups. The DEGs from D6×D7 and reciprocal F_1 hybrids were placed under 36 functional subgroups. In the GO terms of biological processes, a higher percentage of genes were placed in the oxidation-reduction process, transcriptional regulation and gene regulation. For the GO terms of molecular functions, more genes were placed in the ligation and catalytic activities categories. For the GO terms of cell component classification, more genes were placed in the cell and cellular organelles groups. More in-depth analysis of the DEGs in the GO terms in biological pathways was conducted in D6×D7 and reciprocal F_1 hybrids (D7×D6). These GO terms provided important clues as to what biological pathways would play a significant role in heterosis in the direct and reciprocal crosses. Among the several important GO terms, such as oxidation-reduction, transcriptional regulation, protein folding, fungal defense reaction, superoxide reactions, and heat and cold responses were all enriched in the hybrids. These results indicate both D6×D7 and reciprocal F_1 may use the same biological pathways to maintain leaf functions. On the other hand, several significantly differentially expressed GO terms were also found in the two hybrids, such as electron transport chains, photorespiration, regulation of plant hyper-sensitivity and MAPK signal pathways. These biological processes were enriched only in D6×D7.

Discussion

Heterosis has been widely used in crop breeding, and plays an important role in agriculture. However, to this point, the molecular and hereditary mechanisms underlying the heterosis phenomenon is not well understood. Research indicates that the differential gene expression between hybrids and parents may be responsible for heterosis [23]. In over 50 years exploring pepper heterosis, phenotypes [12, 24, 25], physio-biochemical properties [9], heredity and molecular markers [2, 18, 19, 29, 36] have been investigated. Studies associating gene expression to heterosis have gained impressive progress in rice [8, 37, 39], maize [4, 14, 17] and *Arabidopsis* [5]. This is the first study to attempt to identify the relationship between transcriptome expression and heterosis in pepper. RNA-seq analysis of the direct and reciprocal F_1 hybrids generated 0.44 billion high qualities 100 bp reads.

Complementation contributes to transcriptome complexity in maize (*Zea mays* L.) hybrids relative to their inbred parents [17]. Single parent expression, a special instance of the complementation model, can be observed in the reciprocal hybrid in maize [14], rice [39]. In this study, the transcriptome from the reciprocal F_1 (D7×D6) was found to contain 17,096 gene transcripts, which is 120 genes less than D6×D7. Under the single parent expression type, 1,068 (6.20%) and 780 (4.56%) genes were identified in D6×D7 and reciprocal F_1 hybrids. Previous studies suggested a significant positive correlation between gene expression pattern in the "expressed in only one parent" and wheat heterosis [33], and gene expression in the "expressed in one parent and hybrid" with yield traits in cotton [35]. In the four pepper genotypes compared in this study, the following three types of gene expression types, "genes the presence/deletion variations", "expressed in one parent and hybrid" and "expressed in only one parent", belong to the dominant model. Genes enriched in these metabolic pathways should be chosen as priority candidates for studying heterosis.

It was found that hybrids would express a higher ratio of genes following the non-additive expression pattern in studies of maize [14], alfalfa [15], *Larix* [13]. In cases where the non-additive expression type was found to play a role in heterosis formation, it was believed that the super-dominance expression type contributes to the heterosis in the hybrids [3, 15]. Among the four hot pepper genotypes, the ratio of genes belonging to the super-dominance expression type accounts for the largest percentage of transcriptomes, which accounts for 66.08% and 62.96% of genes in the direct and reciprocal hybrids, respectively. In the F_1 hybrids, among all the super-dominance expressed genes, more genes expressed following the positive than the negative super-dominance expression types. Similar gene expression patterns have been found in *Arabidopsis* [5], alfalfa [15], rice [8, 10, 38], maize [27, 28, 31].

In this study, the gene expression levels in the four genotypes should be considered as quantitative traits. When comparing the hybrids to the parents, the expression patterns were divided into three major and seven minor groups. The partial-dominance expression type is the prominent type, which suggests that the additive effect of elite dominant genes in hybrids also contributes to the heterosis. These results concur with the conclusions from a similar study on rice [37]. Compared to the parental inbred lines D6 and D7, fewer gene expression differences were observed between the reciprocal hybrids D6×D7 and D7×D6 harboring identical nuclear genomes, as shown in Fig. 2B. Similar tendencies have been reported in maize [26] and *Arabidopsis* [32], while larger reciprocal effects than in the present study have been documented in rice [8] and maize [28]. The four pair-wise comparisons of gene expression in inbred lines versus hybrids revealed intermediate numbers of differentially expressed genes with reference to the hybrid and inbred line comparisons. Hence, the degree of genomic difference correlates with differential gene expression [34]. In this study, the

number of DEGs between the hybrids and material parent is higher than that from the paternal parent. These results match the conclusion that the material parent has a larger influence than the paternal parent on the heterosis in the hybrids [17].

Only a small percentage of DEGs were identified when compared between the parents and the hybrids, indicating that only a small number of heterosis genes have expressed at different levels from parents to F_1 hybrids. Further study is needed to gain a more in-depth understanding of the functions of these DEGs in heterosis. In this study, DEGs were enriched to different functional GO terms, and these biological pathways were embedded with information that would associate gene expression in the heterosis in D6×D7 and reciprocal F_1 hybrids. Several important enriched GOs in D6×D7 and reciprocal F_1 hybrids matched the pathways identified in other species such as transcriptional regulation in rice [38], and defense and abiotic stress responses in super-rice [6]. However, several GO terms were identified to differ significantly between D6×D7 and reciprocal F_1 hybrids, which include electron transport, photorespiration, and regulation of plant hyper-sensitive response sand MAPK pathways. These pathways were enriched only in D6×D7. These GO terms should be investigated in future studies.

Conclusion

An extensive transcriptome dataset was obtained by RNA-seq, giving a comprehensive overview of the leaf transcriptomes in inbreds and their reciprocal hybrids. Our results provide a useful resource for the hot pepper research community. Using the comparative transcriptome analysis, we detected DEGs. In summary, this present study could provide a series of significant new insights to further explore and understand the formation of heterosis in hot pepper.

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